The EMBO Journal vol.10 no.10 pp.2855-2865, 1991

Functional domains of the granulocyte colony-stimulating factor receptor

Rikiro Fukunaga, Etsuko Ishizaka-Ikeda, Cai-Xi Pan, Yoshiyuki Seto and Shigekazu Nagata

Osaka Bioscience Institute, 6-2-4 Furuedai, Suita-shi, Osaka 565, Japan

Communicated by C.Weissmann

The granulocyte colony-stimulating factor (G-CSF) receptor has a composite structure consisting of an immunoglobulin(Ig)-like domain, a cytokine receptor-homologous (CRH) domain and three fibronectin type III (FNIII) domains in the extracellular region. Introduction of G-CSF receptor cDNA into IL-3-dependent murine myeloid cell line FDC-P1 and pro-B cell line BAF-B03, which normally do not respond to G-CSF, enabled them to proliferate in response to G-CSF. On the other hand, expression of the G-CSF receptor cDNA in the IL-2-dependent T cell line CTLL-2 did not enable it to grow in response to G-CSF, although G-CSF could transiently stimulate DNA synthesis. Mutational analyses of the G-CSF receptor in FDC-P1 cells indicated that the N-terminal half of the CRH domain was essential for the recognition of G-CSF, but the Ig-like, FNIII and cytoplasmic domains were not. The CRH domain and a portion of the cytoplasmic domain of about 100 amino acids in length were indispensable for transduction of the G-CSF-triggered growth signal.

Key words: cytokine receptor family/granulocyte colony-stimulating factor/hemopoietic cells/signal transduction

Introduction

The proliferation and differentiation of hemopoietic cells such as granulocytes, macrophages, T cells and B cells are regulated by a family of cytokines including colony-stimulating factors (CSFs) and interleukins (ILs) (reviewed in Metcalf, 1989). Granulocyte colony-stimulating factor (G-CSF) is a glycoprotein of 174 (human G-CSF) or 178 (murine G-CSF) amino acids, and specifically stimulates colony formation of neutrophilic granulocytes from bone marrow cells (Nagata *et al.*, 1986a,b; Tsuchiya *et al.*, 1986). G-CSF also stimulates proliferation of murine and human myeloid leukemia cells such as NFS-60 (Weinstein *et al.*, 1986), AML-193 (Santoli *et al.*, 1987) and OCI/AML 1 (Nara *et al.*, 1990) cells, while murine WEHI-3B D^+ and 32DC13 cells can be induced by G-CSF to differentiate into granulocytes or monocytes (reviewed in Nicola, 1989; Nagata, 1990). G-CSF produced by stroma cells in bone marrow seems to play an essential role in maintaining the number of neutrophilic granulocytes in peripheral blood. G-CSF is produced also by macrophages stimulated with endotoxins (Metcalf and Nicola, 1985; Nishizawa and Nagata, 1990), and by fibroblasts or endothelial cells treated with TNF-α or IL-1 (Seelentag *et al.*, 1987; Koeffler *et al.*, 1988). The G-CSF thus accumulated in blood appears to be responsible for granulocytosis during the inflammatory process. Since administration of G-CSF to animals caused a marked granulopoiesis (Tsuchiya *et al.*, 1987), the substance is currently under clinical trials for patients suffering from granulopenia (Morstyn *et al.*, 1989).

Despite the biological importance of G-CSF, its mechanism of signal transduction has not been elucidated. A single class of high affinity receptor for G-CSF [dissociation constant (K_d) = 100-500 pM] is present on the precursor and mature cells of neutrophilic granulocytes as well as myeloid leukemia cells (Nicola and Peterson, 1986; Park *et al.*, 1989; Fukunaga *et al.*, 1990b). Purification of the G-CSF receptor from mouse myeloid leukemia NFS-60 cells has indicated that the receptor has an M_r of 100 000-130 000, and the monomeric form of this protein binds G-CSF with a low affinity (K_d = 2.6-4.2 nM), while its oligomeric forms show high affinity binding to G-CSF (K_d = 120-360 pM) (Fukunaga *et al.*, 1990b). Recently, we have isolated the cDNAs for murine (Fukunaga *et al.*, 1990a) and human G-CSF receptors (Fukunaga *et al.*, 1990c); expression of these receptor cDNAs in monkey COS cells gave rise to proteins which specifically bound G-CSF with a high affinity, suggesting that the single polypeptide encoded by the cDNA is sufficient to constitute the high affinity binding site for G-CSF.

Murine and human G-CSF receptors consist of 812 and 813 amino acids, respectively, and contain a single transmembrane domain (Fukunaga *et al.*, 1990a,c). In agreement with the fact that G-CSF has no species specificity between human and mouse, the amino acid sequences of human and murine G-CSF receptors have a considerable similarity (62.5% identity) (Fukunaga *et al.*, 1990c). In the extracellular domain (~600 amino acids) of the G-CSF receptor, there is a region of ~200 amino acids which shows significant homology to the receptors for IL-3-7, erythropoietin and GM-CSF, and the β-chain for the IL-2 receptor (Bazan, 1990a). In the case of the G-CSF receptor, this region (referred to as the cytokine receptor-homologous domain or CRH domain) is followed by a domain (~300 amino acids) consisting of three fibronectin type III (FNIII) modules, which is homologous to chicken contactin (Fukunaga *et al.*, 1990a). The cytoplasmic region of the G-CSF receptor has a limited similarity to that of the IL-4 receptor (Mosley *et al.*, 1989), and like other members of the cytokine receptor family, the region does not appear to contain the domain with kinase or other enzymatic activity. Very recently, the human cDNA for the IL-6 signal transducer, gp130, was isolated (Hibi *et al.*, 1990). The overall structure of gp130 is remarkably similar to that of the G-CSF receptor, and the similarity of human G-CSF receptor and gp130 is 46.3% on the amino acid sequence level, when conservative substitutions are included.

In order to investigate the role of the G-CSF receptor in the G-CSF-dependent signal transduction, we have expressed

the G-CSF receptor cDNA in various hematopoietic cell lines using a promoter of human elongation factor 1α gene. The G-CSF receptor could function as a transducer for the G-CSF-triggered growth signal in IL-3-dependent FDC-P1 and BAF-B03 cells, but not in IL-2-dependent CTLL-2 cells. Utilizing this expression system, we have identified the functional domains of the G-CSF receptor.

Results

Establishment of cell lines expressing the G-CSF receptor

Mouse myeloid precursor cell line FDC-P1 and pro-B cell line BAF-B03 require IL-3 for their growth, whereas the growth of murine cytotoxic T cell line CTLL-2 is strictly dependent on IL-2. In order to examine whether the cloned G-CSF receptor can transduce the G-CSF-triggered signal in these cells, we have established transformed cell lines expressing the G-CSF receptor cDNA. For this purpose, we took advantage of a recently constructed mammalian expression vector, pEF-BOS, which utilizes a constitutive promoter of the human polypeptide chain elongation factor 1α (EF-1α) gene (Uetsuki *et al.*, 1989), and which works very efficiently in various cell lines (Mizushima and Nagata, 1990). The pEF-BOS plasmid harboring a murine G-CSF receptor cDNA (pI62) (Fukunaga *et al.*, 1990a) or human G-CSF receptor cDNA (pHQ3) (Fukunaga *et al.*, 1990c) was transfected into FDC-P1, BAF-B03 and CTLL-2 cells,

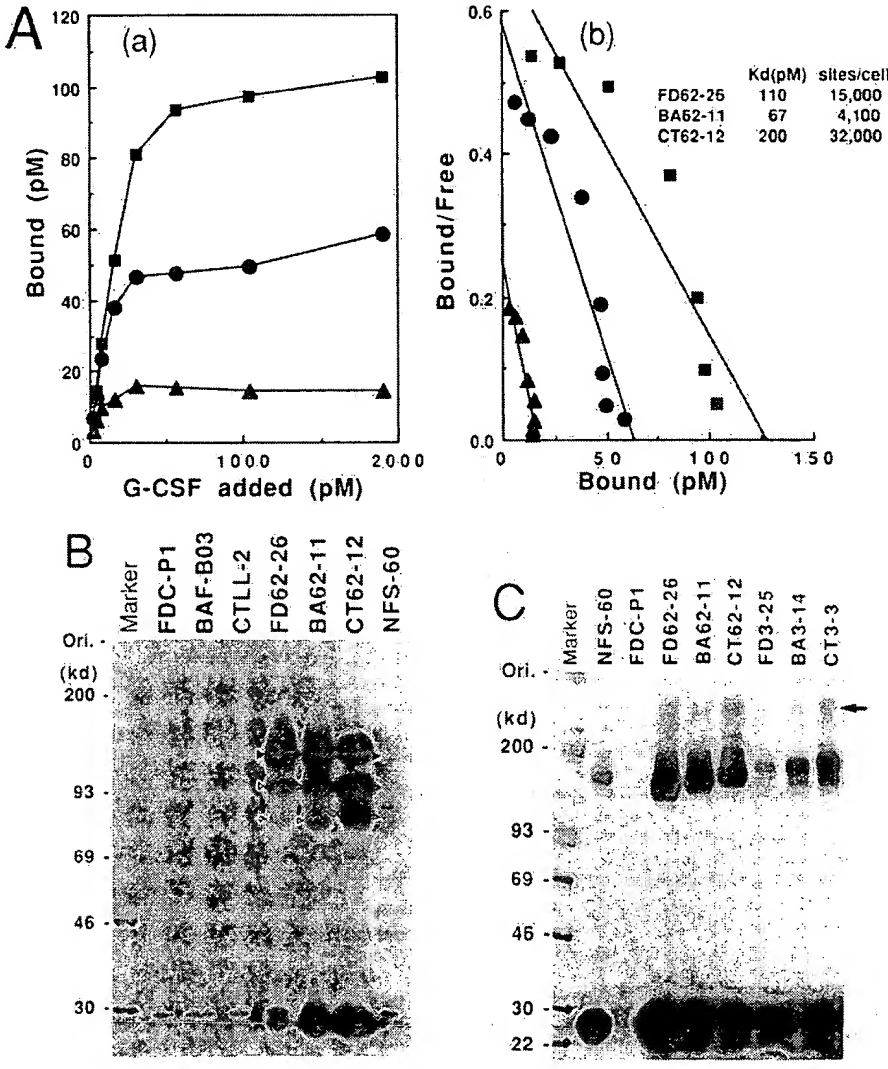


Fig. 1. Expression of mouse and human G-CSF receptor in hemopoietic cell lines. (A) G-CSF binding characteristics in transformant clones. (a) Saturation binding of [^{125}I]G-CSF to transformant clones expressing mouse G-CSF receptor cDNA. The transformants derived from FDC-P1, BAF-B03 and CTLL-2 were designated FD62, BA62 and CT62, respectively. 2.5×10^6 cells/ml were incubated for 3 h at 4°C with various amounts of [^{125}I]G-CSF. The specific binding of [^{125}I]G-CSF to FD62-26 (●), BA62-11 (▲) or CT62-12 (■) was determined as the difference between total binding and non-specific binding which was measured in the presence of 500 nM unlabeled G-CSF. (b) Scatchard plot of G-CSF binding data shown in (a). (B) Immunoblotting of mouse G-CSF receptor with anti-MR9 serum. Cell lysates prepared from parental cell lines, their transformant clones and NFS-60 were analyzed by immunoblotting with an anti-mouse G-CSF receptor serum (anti-MR9) as described in Materials and methods. As size markers, ^{14}C-labeled molecular weight standards (rainbow marker, Amersham) were electrophoresed in parallel ('Marker' lane). The positions of the mature and less glycosylated G-CSF receptors are indicated by solid and open arrowheads, respectively. (C) Chemical cross-linking of the mouse and human G-CSF receptors with radioiodinated G-CSF. NFS-60, FDC-P1 and transformed cell clones (2×10^7 cells/ml) expressing mouse (FD62-26, BA62-11 and CT62-12) or human (FD3-25, BA3-14 and CT3-3) G-CSF receptor cDNA were incubated for 3 h at 4°C with 500 pM [^{125}I]G-CSF and chemically cross-linked with disuccinimidyl suberate and disuccinimidyl tartarate as previously described (Fukunaga *et al.*, 1990b). The cell lysate (50 μg protein) was analyzed by SDS−PAGE, and the gel was dried and exposed to X-ray film.

together with a plasmid carrying the neomycin resistance gene. G418-resistant transformants were tested for the ability to bind [^{125}I]G-CSF, and several independent clones were isolated after limiting dilution from the binding-positive transformants.

The properties of the G-CSF binding to these stable transformants were first examined. The parental FDC-P1, BAF-B03 and CTLL-2 cells did not bind G-CSF (data not shown). On the other hand, the cell clones of FD62, BA62 and CT62, transformed with the mouse G-CSF receptor cDNA, bound G-CSF with high affinities (K_d = 110, 70 and 200 pM, respectively) (Figure 1A). The numbers of the G-CSF receptor expressed in clones of FD62 and BA62 were constantly 4000–15 000 sites per cell, while clones of C-T62 expressed the mouse G-CSF receptor in the range 20 000–30 000 sites per cell. The transformants with the human G-CSF receptor cDNA (FD3, BA3 and CT3 cells) also expressed only a high affinity G-CSF receptor (K_d = 170–300 pM) at 2400–20 000 sites per cell (data not shown). These K_d values are consistent with our previous results obtained with COS cells (Fukunaga *et al.*, 1990a,c) and confirm that the single polypeptide coded by the cloned G-CSF receptor cDNA is sufficient to constitute a high affinity binding site for G-CSF.

For the immunological detection of the G-CSF receptor, two proteins containing a portion of the extracellular domain (MR1, amino acids 36–326) or cytoplasmic domain (MR9, amino acids 631–812) of the mouse G-CSF receptor were produced in *Escherichia coli*, and polyclonal antibodies against these proteins were prepared in rabbits. An immunoblot using anti-MR9 antiserum identified three molecular species varying in apparent molecular size (125–135 kDa, 105–110 kDa and 85–90 kDa) in the transformant clones as well as in mouse NFS-60 cells which express the endogenous G-CSF receptor (Figure 1B). The bands detected at ~30 kDa in the transformant clones are probably degraded forms of the receptor. The 125–135 kDa species seems to be the mature, cell surface receptor because the cross-linking of the cell surface receptor with radioactive G-CSF gave only a ligand–receptor complex with an apparent M_r of 150–160 kDa (Figure 1C). The other two species are likely to be less glycosylated or non-glycosylated intermediates. The small difference in molecular sizes of the receptor (Figure 1B and C) among the cell types could be due to difference in their glycosylation. Cross-linking experiments also confirmed that the transformant clones FD3-25, BA3-14 and CT3-3 expressed human G-CSF receptor (Figure 1C). We have often observed an additional cross-linked complex with higher molecular size in cells expressing a large number of G-CSF receptors (Figure 1C, indicated by an arrow). Since this complex has an M_r of ~300 kDa, it may represent the cross-linked dimer of the G-CSF receptor with which [^{125}I]G-CSF was cross-linked. The immunoblot analysis sometimes showed a faint band at 125–135 kDa in the parental FDC-P1, BAF-B03 and C-TLL-2 cells (Figure 1B). This band seems to be a non-

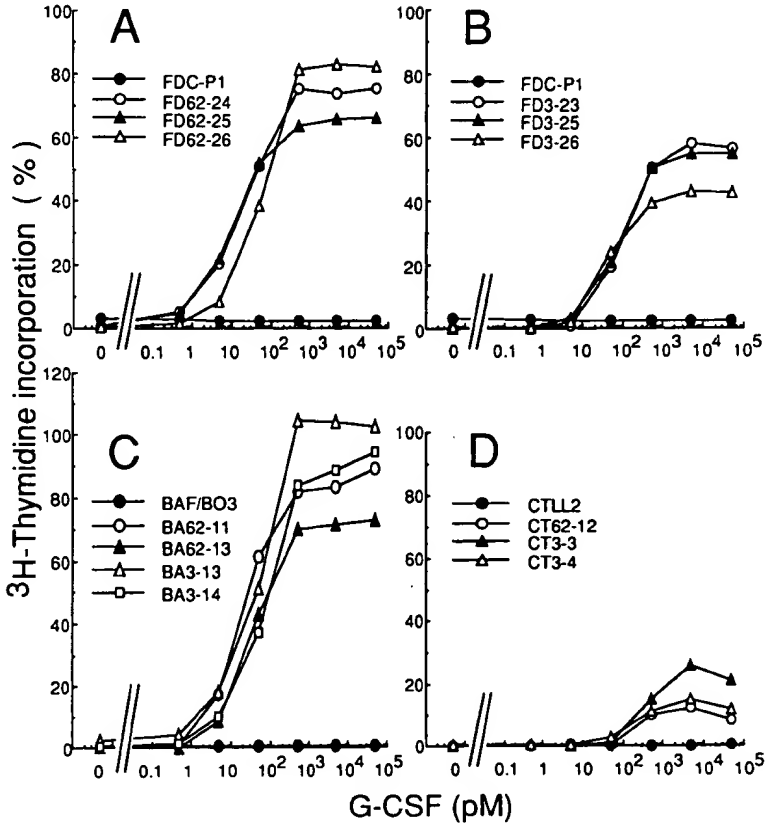


Fig. 2. G-CSF-dependent DNA synthesis of transformants expressing mouse or human G-CSF receptor cDNA. Parental cells and their transformed cell clones expressing mouse or human G-CSF receptor were cultured with 0–50 nM mouse G-CSF, and incorporation of [^{3}H]thymidine into cells was measured. The results are presented as the percentage of the maximum [^{3}H]thymidine incorporation observed with IL-3 (A, B and C) or IL-2 (D) in the respective cells. (A and B) FDC-P1 and its transformant clones expressing the mouse (A) or human (B) G-CSF receptor cDNA. (C) BAF-B03 and its transformant clones expressing mouse or human G-CSF receptor. (D) CTLL-2 and its transformant clones expressing mouse and human G-CSF receptor.

specific one because these cells neither bound G-CSF nor gave the cross-linked complex with [^{125}I]G-CSF (Figure 1C, data not shown).

G-CSF receptor can transduce the growth signal in FDC-P1 and BAF-B03 cells but not in CTLL-2 cells

The effect of G-CSF on the growth properties of the transformants expressing the G-CSF receptor was then examined by [^{3}H]thymidine uptake assay. As shown in Figure 2, none of the parental cell lines responded to G-CSF, whereas transformants derived from FDC-P1 and BAF-B03 responded to G-CSF in a concentration-dependent manner. In the FDC-P1 transformants, clones expressing murine G-CSF receptor (clones of FD62) responded slightly more efficiently than the clones expressing human G-CSF receptor (clones of FD3). The maximum [^{3}H]thymidine uptakes of FD62 and FD3 obtained at >500 pM G-CSF were 70–80% and 50–60%, respectively, of that observed with an excess of murine IL-3 (Figure 2A and B). In BAF-B03 transformants, human and murine G-CSF receptors were equally effective, and the maximum response obtained at 500 pM of G-CSF was almost comparable to that observed with mouse recombinant IL-3 (Figure 2C). In accordance with the high responsiveness of these transformants to G-CSF, 500 pM G-CSF could support the long-term growth of clones of FD62, FD3, BA62 and BA3 in the absence of IL-3 (Figure 3). On the other hand, G-CSF stimulated very weakly the DNA synthesis of the CTLL-2 transformants (clones of CT62 and CT3) (Figure 2D). The response was <30% of that observed with an excess of IL-2, and this weak responsiveness was insufficient to support the long-term proliferation or survival of these cells in the absence of IL-2 (Figure 3).

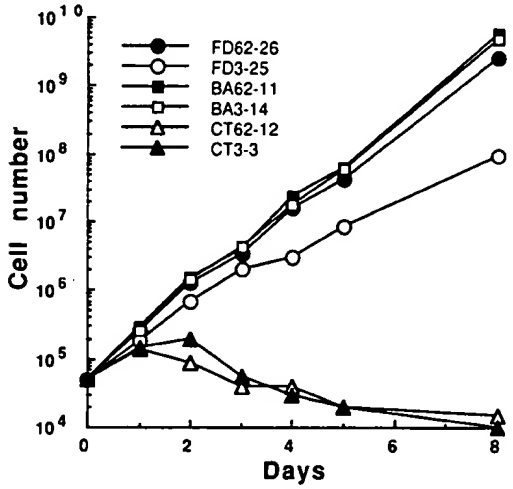
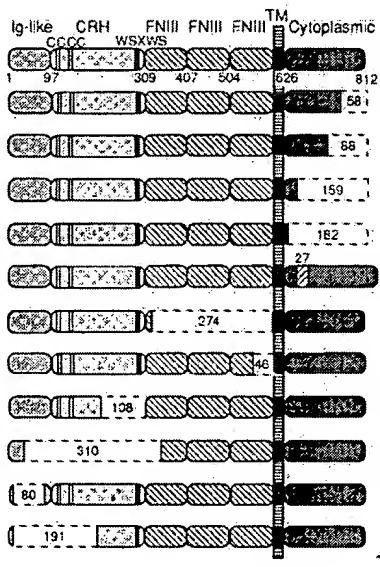


Fig. 3. G-CSF-dependent long-term growth of transformants expressing G-CSF receptor cDNA. Transformants growing in IL-3 or IL-2 were washed thoroughly and cultured at 5×10^4 cells/ml in RPMI1640 containing 10% FCS and 500 pM mouse G-CSF. Cell numbers were counted daily and cell concentrations were kept less than 10^6 cells/ml by appropriate dilution with the same medium.

Construction and expression of cDNAs carrying various mutations in the subdomains of the G-CSF receptor

As shown in Figure 4, the G-CSF receptor contains a single transmembrane region which divides the molecule into two regions, the extracellular and the cytoplasmic. The extracellular region can be further divided into five subdomains, an immunoglobulin(Ig)-like domain, a CRH domain and three FNIII domains, based on homology with other proteins (Fukunaga *et al.*, 1990a; Bazan 1990a) and the exon–intron organization of the G-CSF receptor gene (Y.Seto, R.Fukunaga and S.Nagata, unpublished results).



Mutant	G-CSF binding Kd (pM)	G-CSF binding sites/cell	G-CSF-dependent growth
Wild type	203 ± 87	5500 ~ 15000	+++
A Δ(755-812)	232 ± 28	9300 ~ 13000	+++
E Δ(725-812)	178 ± 19	4800 ~ 6700	++
P Δ(654-812)	207 ± 33	3200 ~ 7400	-
X Δ(631-812)	174 ± 43	1500 ~ 6200	-
11 Ins27(658)	182 ± 39	3300 ~ 11000	+
C Δ(324-597)	475 ± 71	1100 ~ 2300	+
7 Δ(553-598)	175 ± 20	350 ~ 820	+
17 Δ(201-308)	11000 ± 1600	8300 ~ 8400	-
W Δ(37-346)	-	no binding	-
G Δ(5-84)	3650 ± 180	2100 ~ 4100	+
Y Δ(5-195)	-	no binding	-

Fig. 4. Summary of mutational analysis of the G-CSF receptor. The extracellular domain of the G-CSF receptor is putatively divided into five subdomains, an Ig-like domain, a cytokine receptor-homologous (CRH) domain and three fibronectin type III (FNIII) domains (top). Four cysteine residues and a WSXWS motif conserved in the CRH domain are also indicated. The deletion mutants of mouse G-CSF receptor (mutants A, E, P, X, C, W, G and Y) and three variants (variants 11, 7 and 17), are shown schematically below the wild-type receptor. The portions deleted in the mutants are represented by the dotted lines and the number of amino acids deleted in each mutant is indicated. In all wild-type and mutant cDNAs, the coding region for the mature protein is preceded by an N-terminal signal sequence, which is not shown in the figure. The table at the right shows the G-CSF binding characteristics of the mutated receptor and their abilities to transduce the G-CSF-triggered growth signal.

In order to assign the functional domains of the G-CSF receptor, we have constructed a series of murine G-CSF receptor cDNAs which have various deletions in these subdomains. In the first set of deletion mutants (mutants A, E, P and X), the cytoplasmic region of the receptor was progressively deleted from the C-terminal end up to amino acid positions 754, 724, 653 and 630, respectively (Figure 4). For the extracellular region, four deletion mutants (C, W, G and Y) were constructed. In addition to these constructions, we have used as mutants variant receptors whose cDNAs were isolated from human and mouse cDNA libraries. The human G-CSF receptor variant (mutant 11) contains a 27 amino acid insertion between amino acid positions 657 and 658 in the cytoplasmic domain (Fukunaga *et al.*, 1990c), while murine variants carry the deletion of the C-terminal half of the third FNIII domain (mutant 7) or the C-terminal half of the CRH domain (mutant 17) (Figure 4). These G-CSF receptor variants seem to be generated by alternative splicing of the precursor RNA (Y.Itoh, E.Ishizaka-Ikeda, Y.Seto, R.Fukunaga and S.Nagata, unpublished results).

FDC-P1 cells were transformed with expression plasmids carrying the mutated cDNA as described above. Stable transformants expressing each mutant were isolated and designated FD-A, FD-E, FD-P and so forth. Expression of the mutated receptor in these transformants was first analyzed by immunoblot analysis using anti-MR9 antiserum. As shown in Figure 5A, the mature and less glycosylated receptor with molecular weights expected from the size of the deletion was observed in all transformant clones except for clones of FD-P and FD-X, which were transformed with mutants containing little or no part of the MR9 polypeptide. A relatively weak intensity observed in mutants A and E was also due to deletion of parts of the cytoplasmic domain. An immunoblot with anti-MR1 antiserum recognizing a part of the extracellular domain of the mouse G-CSF receptor showed bands in transformant clones for all mutants except for the mutant W which had a deletion of the region recognized by anti-MR1 serum (Figure 5B). Furthermore, as shown in Figure 5C, cross-linking with [^{125}I]G-CSF gave a complex of the expected size in all transformant clones which bind G-CSF with a high affinity (Figure 4). In contrast, no cross-linked complex was observed in clones FD-17 (Figure 5C, lane 6), FD-G, FD-W and FD-Y (data not shown) which have a low or undetectable affinity to G-CSF (Figure 4). Expression of a variant of the human G-CSF receptor in FD11-10 cells was confirmed by the cross-linking experiment (Figure 5C). These results indicate that all mutants of the G-CSF receptor were expressed in the transformants, although the expression levels of mutants C, 7 and G, which have deletions in the FNIII or Ig-like domain, were relatively low in all clones so far tested (Figures 4 and 5).

The CRH domain is responsible for binding of G-CSF

Using the FDC-P1 transformants expressing the mutated G-CSF receptor, we have located the region of the receptor essential for binding of the ligand. Typical Scatchard plots of the G-CSF binding data with these transformants are shown in Figure 6, and summarized in Figure 4. The mutant

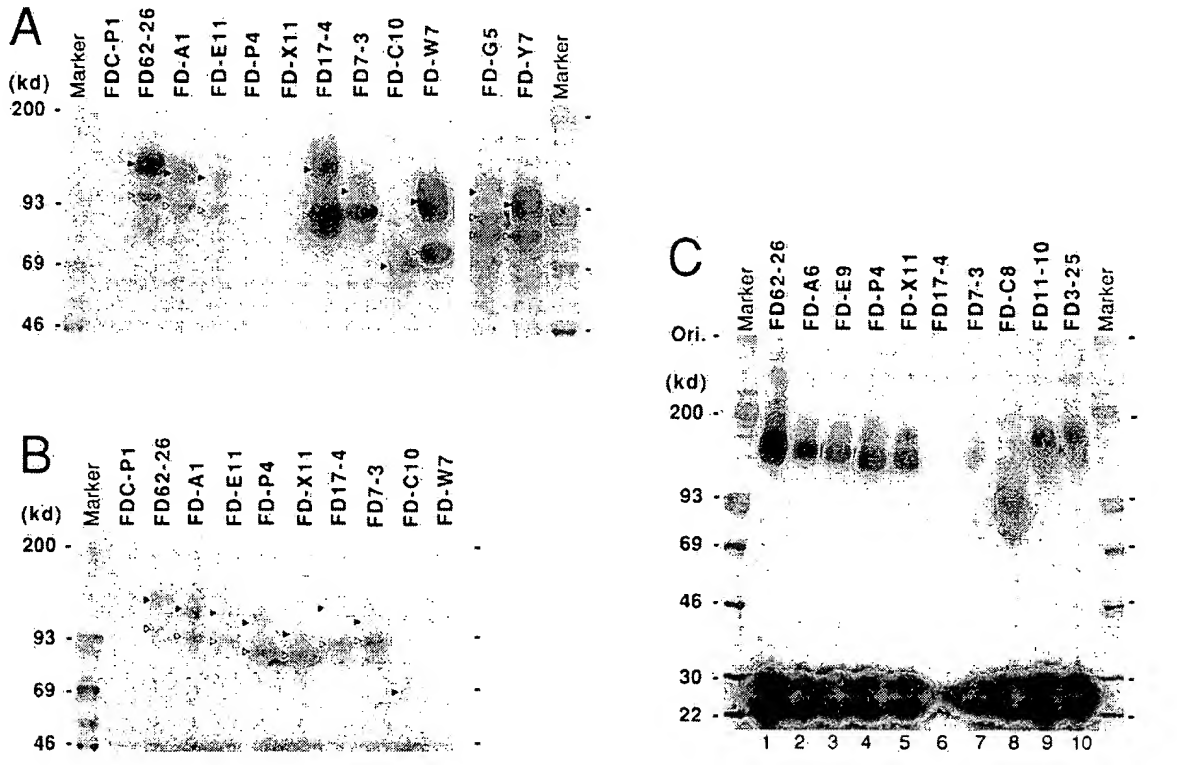


Fig. 5. Expression of G-CSF receptor mutants in FDC-P1 transformants. (A and B) Cell lysates of FDC-P1-derived transformants expressing wild-type or mutant cDNA of mouse G-CSF receptor were analyzed by immunoblotting with the anti-MR9 antiserum (A), or anti-MR1 antiserum (B) as described in Figure 1B. (C) The wild-type and mutant G-CSF receptors expressed in FDC-P1 transformants were cross-linked with [^{125}I]G-CSF and analyzed as described in Figure 1C. The cell lysate of 50 μg (lanes 1–5), 200 μg (lanes 6–8) or 100 μg (lanes 9 and 10) protein was electrophoresed.

X, containing only five amino acids in the cytoplasmic region, as well as mutants A, E, P and 11, could bind G-CSF with the same affinity as the wild-type receptor (K_d ~ 200 pM). These results indicate that the truncations or mutations in the cytoplasmic region do not affect the extracellular binding of G-CSF to the receptor.

Two mutants containing deletions in the FNIII domains (mutants 7 and C) could bind G-CSF with high affinities, although mutant C showed slightly lower affinity (K_d = 480 pM) than the wild-type receptor. On the other hand, mutant 17, which lacks the C-terminal 108 amino acids of the CRH domain, and mutant G, which lacks the Ig-like domain, bound G-CSF with ~50- and 20-fold higher K_d values (K_d = 11.0 and 3.7 nM), respectively, than the wild-type receptor (Figures 4 and 6). The deletion either in the N-terminal half or in the entire region of the CRH domain (mutants Y and W) resulted in total inability to bind G-CSF. These results indicate that the N-terminal region of the CRH domain plays an essential role in the binding of G-CSF.

The CRH domain and part of the cytoplasmic region are necessary for signal transduction

We have then examined whether mutated G-CSF receptors are able to transduce the G-CSF-triggered growth signal into cells. Figure 7A shows the G-CSF dependent [^{3}H]thymidine uptake of the transformants expressing the mutated G-CSF receptor. Clones of FD-A and FD-E responded to G-CSF in a concentration-dependent manner, although the maximum response of FD-E clones was lower than that obtained with the wild-type G-CSF receptor. Clones of FD-A and FD-E could be maintained in the medium containing G-CSF instead of IL-3 (Figure 7B). On the other hand, G-CSF neither stimulated the DNA synthesis of FD-P and FD-X clones nor supported their long-term growth (Figure 7A and B). These results suggest that the 99 amino acid portion from position 626 to 724 in the cytoplasmic region is essential for transduction of the G-CSF-triggered growth signal into FDC-P1 cells. FD11 clones expressing the molecule which contains a 27 amino acid insertion responded weakly to G-CSF, and were able to grow slowly in the medium containing G-CSF.

G-CSF weakly stimulated [^{3}H]thymidine uptake of FD-7, FD-C and FD-G clones (Figure 7A) and these clones could be maintained in the medium containing G-CSF (Figure 7B), suggesting that neither Ig-like nor FNIII domains are involved directly in signal transduction. As expected from the total inability of the mutants W and Y to bind G-CSF, these receptors could not respond to G-CSF at all. It may be noteworthy that a weak [^{3}H]thymidine uptake independent of G-CSF and IL-3 was observed in some clones of FD-C, FD-G and FD-Y cells (Figure 7A). Interestingly, mutant 17, which contained a deletion in the C-terminal half of the CRH domain, was completely inactive in signal transduction even in the presence of 50 nM G-CSF (Figure 7A), which should be sufficient for the binding of G-CSF to this mutant (Figures 4 and 6). These results suggest that the CRH domain of the receptor plays an essential role in signal transduction by G-CSF.

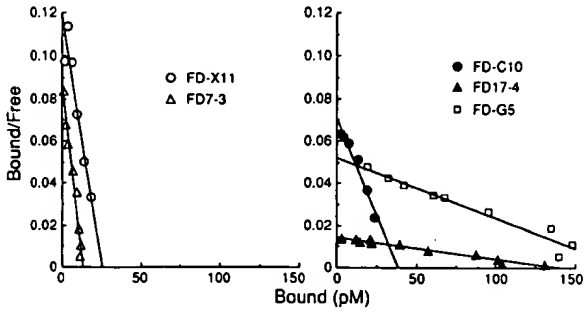


Fig. 6. Scatchard analysis of mutant G-CSF receptors expressed in FDC-P1 transformants. Cells of transformants FD-X11 (2.5×10^6 cells/ml), FD-C10, FD7-3, FD17-4 (10^7 cells/ml) and FD-G5 (2.7×10^7 cells/ml) were incubated with various concentrations of [^{125}I]G-CSF for 3 h at 4°C and specific binding was determined as described in Figure 1A.

Discussion

G-CSF receptor is able to function as a growth signal transducer in IL-3-dependent cells but not in IL-2-dependent cells

To explore the signal transduction mechanism of the newly identified cytokine receptor family (Bazan, 1990b), it is essential to introduce the receptor cDNAs into various cells, especially hemopoietic cells. However, the expression of cDNA in hemopoietic cells is not an easy task. In this report, we have successfully used the promoter of human EF-1α gene to express the G-CSF receptor cDNA in various hemopoietic cell lines (Figure 1). The expression level of the G-CSF receptor driven by the EF-1α promoter in murine FDC-P1 cells was ~100 times greater than that driven by a CMV promoter (unblished observation).

Expression of the G-CSF receptor in IL-3-dependent FDC-P1 or BAF-B03 cells enables these cells to grow in response to G-CSF (Figures 2 and 3). These results clearly indicate that the G-CSF receptor encoded by the cloned cDNA is sufficient to transduce the growth signal into cells, and suggest the presence of a common signal transducing pathway for the IL-3 and G-CSF systems. Introduction of protein kinases such as v-src, v-abl and v-fms into IL-3-dependent cells abrogated the dependence of cells on IL-3 (Cleveland *et al.*, 1989). Stimulation of cells with IL-3 induces the phosphorylation of tyrosine residues in a set of proteins, suggesting that some tyrosine kinase is involved in signal transduction by IL-3 (Koyasu *et al.*, 1987; Isfort *et al.*, 1988). Recently, Isfort and Ihle (1990) have shown that IL-3 and G-CSF stimulate tyrosine phosphorylation of the same protein (pp56) in NFS-60 cells. It is possible that the pp56 protein is one of the signal transducing molecules common in IL-3 and G-CSF systems.

Hatakeyama *et al.* (1989b) introduced the cDNA for the β-chain of IL-2 receptor into IL-3-dependent BAF-B03 cells. Since the BAF-B03 cells expressing IL-2 receptor could grow in the presence of IL-2, these authors postulated common signal transduction pathways in the IL-2 and IL-3 systems. However, G-CSF did not support the growth of the IL-2-dependent CTLL-2 cells transformed with the G-CSF receptor cDNA, though these cells expressed the high affinity G-CSF receptor in a large amount (Figure 1). These results may suggest that the signal transducing pathways downstream of the receptor are similar but different in the G-CSF and IL-2 systems, and that CTLL-2 cells are deficient in some components which are necessary for G-CSF-triggered signal transduction.

Homodimer and heterodimer of the cytokine receptor

As shown in Figure 8, recent analyses on the structure of cytokine receptors and reconstitution of the receptors using their respective cDNAs have revealed that the receptors for IL-6, IL-2 and GM-CSF consist of two different subunits (Hatakeyama *et al.*, 1989a; Hibi *et al.*, 1990; Hayashida *et al.*, 1990). The α-chain of each receptor binds its ligand with low affinity, and the second chain (β-chain) is necessary for formation of the high affinity binding site and for transduction of the signal. In contrast, the single polypeptide of the G-CSF receptor constituted a high affinity binding site for G-CSF, not only in hemopoietic cells (Figure 1A) but also in epithelial cells such as COS cells (Fukunaga *et al.*, 1990a,c) and C127I cells (unpublished observation). Previously, we have shown that the monomer of the purified G-CSF receptor has a low affinity for G-CSF, whereas its dimer or oligomer constitutes a high affinity binding site for G-CSF (Fukunaga *et al.*, 1990b). Since the dissociation

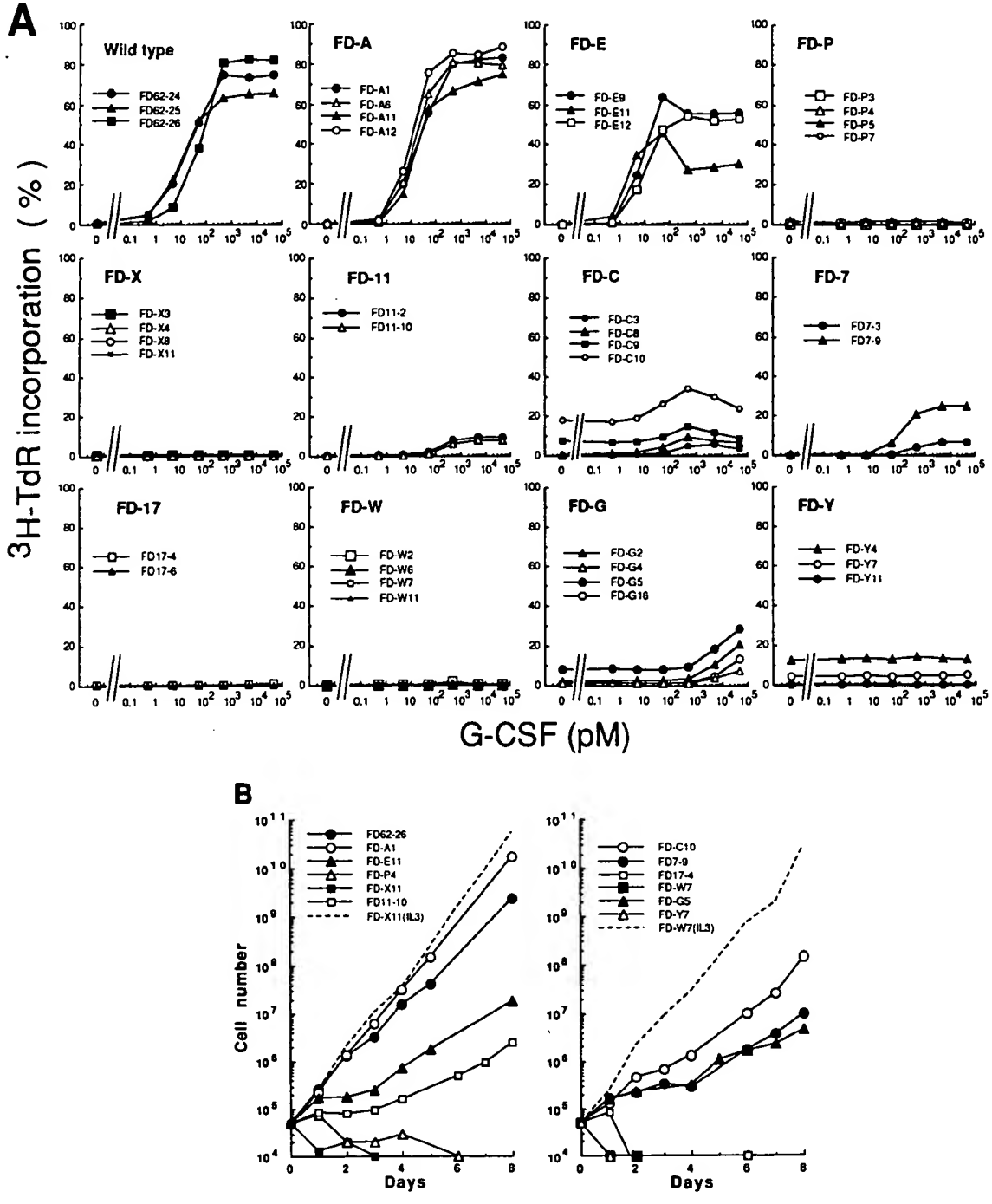


Fig. 7. G-CSF-dependent growth of transformants expressing mutant G-CSF receptor. (A) G-CSF-dependent [^{3}H]thymidine incorporation into cells. [^{3}H]thymidine incorporation was measured with several independent clones of individual FDC-P1 transformants expressing the mutant receptor as described in Figure 2. (B) G-CSF-dependent long-term proliferation of the transformants expressing mutant G-CSF receptor. Increase in cell number of FDC-P1 transformants expressing the mutant receptor in the presence of 20 nM (for FD-G transformant) or 500 pM (for other transformants) G-CSF was counted as described in Figure 3. Dotted lines indicate the growth curve of FD-X11 and FD-W7 cells in the presence of IL-3.

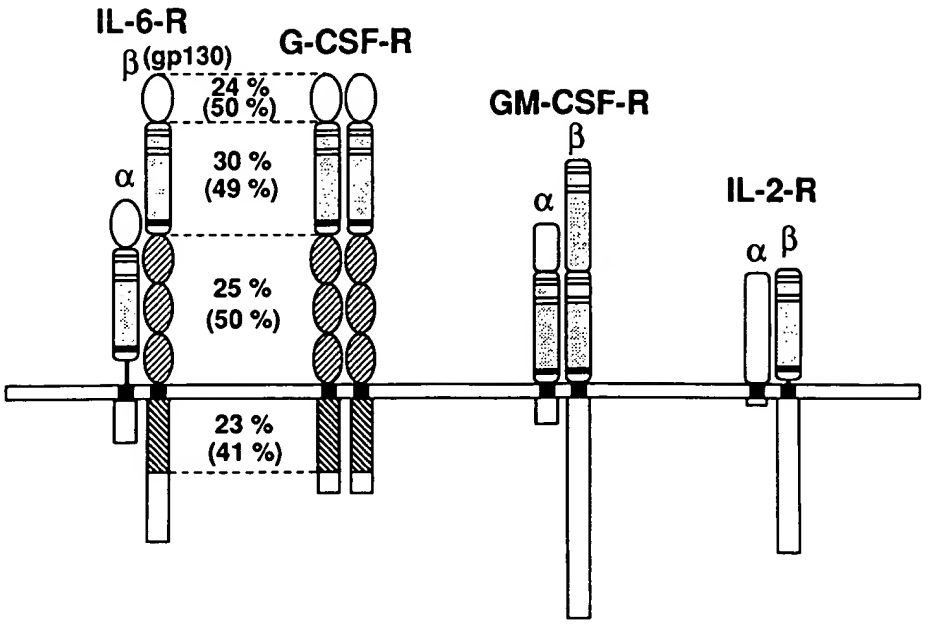


Fig. 8. Schematic representation of homo- and heterodimeric structures of functional, high affinity receptors for G-CSF, GM-CSF, IL-2 and IL-6. The CRH domains containing the conserved cysteine residues (thin bars) and the 'WSXWS' motif (thick bar) are indicated by shaded boxes. The gp130 protein (the β-chain of the IL-6 receptor) shows remarkable homology with the G-CSF receptor except for a C-terminal part of the cytoplasmic domain. The numbers indicate the percentage of identical amino acids in each subdomain. The numbers in parentheses show the percentage of homology including the conservative substitutions.

constant observed in intact cells is similar to that obtained with the dimer of the purified G-CSF receptor, it is likely that the G-CSF receptor exists as a dimer on the cell surface. Indeed, the cross-linking of the receptor with [^{125}I]G-CSF suggested the existence of a dimer of the receptor (Figure 1C). Availability of the antibody against the G-CSF receptor will make it possible to clarify the mechanism of the receptor dimerization in more detail.

Ligand binding domain of G-CSF receptor

Since the IL-4, IL-7 and erythropoietin receptors and the β-chain for the IL-2 receptor contain only the CRH domain in the extracellular region, it was postulated that this domain is involved in binding of the ligand (Bazan, 1990b). The present study using deletion mutants of the G-CSF receptor agrees with the above hypothesis. The receptor deleted in the C-terminal half of the CRH domain (mutant 17) could bind G-CSF with low affinity, while the mutants lacking the N-terminal region of the CRH domain (mutants W and Y) completely lacked the ability to bind G-CSF, suggesting that the N-terminal half containing the four conserved cysteine residues is indispensable for binding the ligand. Recently, Bass *et al.* (1991) have also shown that the hormone binding determinants of the growth hormone receptor is in the cysteine-rich region of the CRH domain.

The fact that mutants G and 17 bound G-CSF with low affinities suggests that the Ig-like domain and/or the C-terminal portion of the CRH domain may participate in the recognition of G-CSF, although it is possible that deletions in these domains simply caused some steric hindrance or conformational change in the ligand-binding site. An alternative possibility is that these domains are responsible for dimerization of the G-CSF receptor. The latter possibility may explain why these mutants showed low affinity binding to G-CSF with K_d values (3.7 and 11 nM) similar to those observed with the monomeric protein of the purified G-CSF receptor (2.6–4.2 nM, Fukunaga *et al.*, 1990b). No cytokines showed a significant homology to G-CSF except for IL-6, which has a limited similarity to G-CSF (Nagata, 1990). Nevertheless, the CRH domain, which is the ligand-binding domain of the cytokine receptor molecule, showed a significant homology among these cytokines (Bazan, 1990b). Bazan (1990a) has recently proposed, based on the extrapolation from their primary structures, that these cytokines may have similar tertiary structure. Detailed mutational analysis of the ligand and the CRH domain, as carried out with growth hormone (Cunningham and Wells, 1989; Bass *et al.*, 1991), may reveal the mechanism through which different cytokines specifically recognize their own receptor.

Signal transducing domain of the G-CSF receptor

In the two mutants showing low affinity binding to G-CSF, the mutant G could respond to high concentrations of G-CSF, whereas the mutant 17 was totally inactive (Figures 4, 6 and 7). This result suggests that the C-terminal region of the CRH domain is indispensable for the signal transduction triggered by G-CSF. This C-terminal region, which contains the 'WSXWS' motif, may play a role in transducing some conformational change induced by the binding of G-CSF across the membrane to the cytoplasmic domain of the molecule. Alternatively, if the region deleted in the mutant 17 is involved in the formation of the dimeric receptor as discussed above, this result may imply that dimerization of the G-CSF receptor has a significance in signal transduction, as suggested in the cases of PDGF and EGF receptors (Heldin *et al.*, 1989; Bishayee *et al.*, 1989; Spaargaren *et al.*, 1991). In the [^{3}H]thymidine incorporation assay, the transformants expressing mutants G, C and 7 responded weakly to G-CSF (Figure 7A) but were able to grow slowly in the presence of G-CSF (Figure 7B). The Ig-like and FNIII domains, therefore, do not seem to play

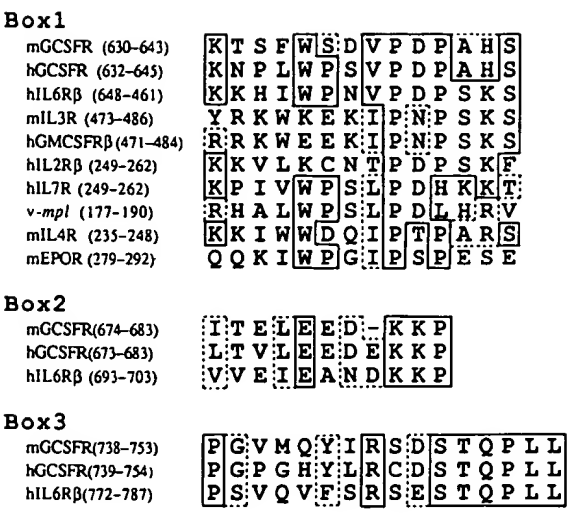


Fig. 9. The amino acid sequences conserved in the cytoplasmic domains of the G-CSF receptor and the β-chain (gp130) of the IL-6 receptor. The amino acid sequences of the corresponding regions of murine G-CSF receptor (mGCSFR, Fukunaga *et al.*, 1990a), human G-CSF receptor (hGCSFR, Fukunaga *et al.*, 1990c) and the β-chain of human IL-6 receptor (gp130) (hIL6Rβ, Hibi *et al.*, 1990) are aligned. The amino acid sequence of the Box 1 region of murine IL-3 receptor (Itoh *et al.*, 1990), the β-chain of human GM-CSFR (Hayashida *et al.*, 1990), the β-chain of human IL-2 receptor (Hatakeyama *et al.*, 1989a), human IL-7 receptor (Goodwin *et al.*, 1990), v-*mpl* (Souyri *et al.*, 1990), murine IL-4 receptor (Mosley *et al.*, 1989) and murine erythropoietin receptor (D'Andrea *et al.*, 1989) are also shown.

essential roles in signal transduction. Binding experiments using [^{125}I]G-CSF and Western blotting analysis have indicated that the numbers of the receptor expressed in these cells are lower than those in cells expressing the wild-type receptor (Figures 4 and 5). These results suggest that deletion of the FNIII domains may render the molecule unstable. The low response of these cells to G-CSF may be partly due to the poor expression of these mutant proteins.

The progressive deletion of the cytoplasmic region of the G-CSF receptor identified a region of 99 amino acids (positions 626−724) which seems to be essential for signal transduction by G-CSF in FDC-P1 cells. This region may constitute a domain of an unknown enzyme activity or associate with other molecules which transduce the signal. Previously, we have noticed that the cytoplasmic region of the G-CSF receptor has a similarity to that of the IL-4 receptor (Fukunaga *et al.*, 1990a). However, the homology between the cytoplasmic regions of the G-CSF receptor and the newly isolated gp130 (the β-chain) of the IL-6 receptor (Hibi *et al.*, 1990) is much more pronounced (Figure 8). As shown in Figure 9, three stretches of amino acid sequences (Boxes 1−3) are highly conserved between the G-CSF receptor and gp130, and two of the sequences (Boxes 1 and 2) are in the region which is critical for the signal transduction of the G-CSF receptor. IL-6 works on various cells including B cells, T cells and myeloid cells, and the gp130 seems to be responsible for the signal transduction triggered by IL-6 (Hibi *et al.*, 1990). The conserved region identified above may also be involved in signal transduction through the gp130 in the IL-6 system. In this regard, it may be noteworthy that the sequences similar to 'Box 1' can be found in the cytoplasmic region of receptors for IL-3, GM-CSF, IL-2, IL-7, IL-4 and erythropoietin, and in a recently identified oncogene v-*mpl* (Figure 9). In addition to the stimulation of cell proliferation, G-CSF and IL-6 have an ability to induce cells to differentiate. Whether or not the region involved in transducing the differentiation signal is identical to the region for the growth signal remains to be studied.

Materials and methods

Cells and cell culture

Murine myeloid leukemia cell lines, NFS-60 cells (Weinstein *et al.*, 1986) and FDC-P1 cells (Dexter *et al.*, 1980) were kindly provided by Dr J.N.Ihle (St Jude Children's Research Hospital). Mouse pro-B cell line, BAF-B03 (Hatakeyama *et al.*, 1989b) and mouse cytotoxic T cell line, CTLL-2 (ATCC TIB 214) were provided by Drs M.Hatakeyama and T.Taniguchi (Institute for Molecular and Cellular Biology, Osaka University). NFS-60, FDC-P1 and BAF-B03 cells were maintained in RPMI1640 medium supplemented with 10% fetal calf serum (FCS, Hyclone) and 10−20 U/ml of recombinant mouse IL-3 (Fukunaga *et al.*, 1990b). CTLL-2 cells were grown in RPMI1640 medium containing 10% FCS and 10 ng/ml recombinant human IL-2 which was kindly provided by Dr H.Matsui (Ajinomoto Co., Tokyo).

Plasmid construction

Plasmid pI62 carrying the full length cDNA for mouse G-CSF receptor in CDM8 vector has been described previously (Fukunaga *et al.*, 1990a). Two variant cDNAs, pG17 and pF1, were obtained from the NFS-60 cDNA library (Fukunaga *et al.*, 1990a) by colony hybridization with the cDNA of pI62 as a probe, and will be described elsewhere in detail. The *Xba*I cDNA fragment was excised from pI62, pG17 and pF1, and inserted into the *Xba*I site of a mammalian expression vector pEF-BOS (Mizushima and Nagata, 1990) to produce pBOS-I62 (wild-type receptor), pBOS-G17 (variant 17) and pBOS-JF7 (variant 7). The expression plasmid for the wild-type human G-CSF receptor (pHQ3) and its variant 11 (pQW11) has been described previously (Fukunaga *et al.*, 1990c).

For the construction of mutants containing the deletion in the cytoplasmic domain of mouse G-CSF receptor, pI62 was digested with either *Xmn*I (at 2140), *Bsp*HI (at 2209), *Bst*EII (at 2421) or *Apa*I (at 2516), and if necessary, ends were blunted with the Klenow fragment of *E.coli* DNA polymerase I or T4 DNA polymerase. An *Xba*I linker (CTCTAGAG), which allows the addition of a leucine residue followed by an in-frame termination codon, was ligated to the blunt-ended DNA and digested with *Xba*I. The *Xba*I fragments containing the G-CSF receptor cDNA were then inserted into pEF-BOS to generate pBOSdXmn (mutant X), pBOSdBsp (mutant P), pBOSdBstE (mutant E), and pBOSdApa (mutant A).

To construct pBOSdWS (mutant W), pBOS-I62 was digested with *Kpn*I (at 1829) and *Bgl*II (at 358), and the *Bgl*II−*Kpn*I DNA fragment derived from the cDNA was digested with *Xho*II (at 1223 and 1288). The *Xho*II−*Kpn*I DNA fragment was separated using agarose gel electrophoresis and inserted into pBOS-I62, which was digested with *Kpn*I (at 1829) and partially digested with *Bgl*II (at 358). For the construction of pBOSdCON (mutant C), pI62 was digested with *Eco*T22I (at 1106) and *Bsp*HI (at 2209), and the *Eco*T22I−*Bsp*HI cDNA fragment was digested with *Xho*II (at 1223, 1288 and 2045). The 117 bp *Eco*T22I−*Xho*II and the 164 bp *Xho*II−*Bsp*HI DNA fragments were then ligated with pI62 digested with *Eco*T22I and *Bsp*HI. The *Xba*I DNA fragment of the resultant plasmid was inserted into pEF-BOS to produce pBOSdCON. To construct pBOSdIg (mutant G), the cDNA fragment of pI62 was digested with *Taq*I (at 265, 838 and 2000), treated with Klenow fragment and digested with *Hin*dIII (at 162) to yield a 103 bp *Hin*dIII−*Taq*I(blunt) fragment. In addition to this fragment, the cDNA fragment of pI62 was digested with *Tth*111I (at 297, 501 and 1938), blunt-ended and digested with *Kpn*I (at 1829). The 103 bp *Hin*dIII−*Taq*I(blunt) fragment and the 1.33 kb *Tth*111I(blunt)−*Kpn*I fragment were ligated together with a 6.3 kb *Hin*dIII−*Kpn*I fragment, which was prepared by digestion of pBOS-I62 with *Kpn*I (at 1829) and *Hin*dIII (partially at 162) to produce pBOSdIg. To construct pBOSdICy (mutant Y), the cDNA fragment of pI62 was digested with *Taq*I (at 265, 838 and 2000), *Hin*dIII (at 162) and *Kpn*I (at 1829); the 103 bp *Hin*dIII−*Taq*I fragment and 991 bp *Taq*I−*Kpn*I fragment were then ligated with the 6.3 kb *Hin*dIII−*Kpn*I fragment described above. All constructions were confirmed by restriction enzyme mapping and DNA sequencing analysis.

Transfection of DNA

Cells were transfected with plasmid DNAs by electroporation (Potter *et al.*, 1984). The electroporation was carried out using Gene Pulser (BioRad) essentially according to the manufacturer's instructions. In brief, 8×10^6 cells were suspended in 0.8 ml of phosphate-buffered sucrose [7 mM sodium phosphate buffer (pH 7.4), 270 mM sucrose and 1 mM $MgCl_2$]. Eighty micrograms of the G-CSF receptor expression plasmid which had been

linearized by digestion with *Apa*LI, and 2 μg of *Xho*I-digested pSTneoB (Katoh *et al.*, 1987) were added to the cell suspension, which was incubated on ice for 10 min. Cells were exposed to a 350 V pulse with a capacitance of 25 μF, and returned to ice. After incubation on ice for 10 min, cells were diluted with 50 ml of RPMI 1640 medium/10% FCS containing IL-3 or IL-2, and cultivated in 24-well plates. Transfected cells were selected by culturing cells in medium containing G-418 at a final concentration of 0.5 mg/ml (for FDC-P1, and CTLL-2) or 2 mg/ml (for BAF-B03). Subcloning of the transfected cells was carried out by limiting dilution.

Cell proliferation assay, binding of G-CSF and chemical cross-linking

1.5×10^4 cells (100 μl) were mixed with various concentrations of G-CSF, IL-3 or IL-2 in 96-well microtiter plates. After incubation at 37°C for 22 h, 0.5 μCi of [^{3}H]thymidine (specific activity, 74 GBq/mmol) was added per well and further incubated for 4 h at 37°C prior to harvest.

Radioiodination of murine recombinant G-CSF, binding of [^{125}I]G-CSF to cells, and chemical cross-linking were performed as described previously (Fukunaga *et al.*, 1990a,b).

Preparation of antibodies against murine G-CSF receptor

Two different anti-mouse G-CSF receptor sera (anti-MR1 and anti-MR9) recognizing either a portion of the extracellular domain (amino acid positions 36–326) or the cytoplasmic domain (positions 631–812) were prepared as follows. The corresponding regions of murine G-CSF receptor were first produced in *E.coli* using the expression system developed by Studier *et al.* (1990). The expression vector pGEMEX-1 (Promega) was digested with *Nhe*I and *Bam*HI and the ends were filled in using the Klenow fragment. The DNA was religated to generate pEX, in which the *phi10* gene was deleted and the *Bam*HI site was placed immediately downstream of an ATG initiation codon. To express the extracellular portions of murine G-CSF receptor, the 865 bp *Xho*II DNA fragment (nucleotide position 358–1223) of pI62 was inserted into the *Bam*HI site of pEX to generate pEX-MR1. To express the cytoplasmic region of murine G-CSF receptor, pGEX-MR9 was constructed by ligating the 900 bp *Xmn*I–*Sph*I DNA fragment (nucleotides 2140–3126) of pJ17 (Fukunaga *et al.*, 1990a) with the *Hinc*II- and *Sph*I-digested pGEMEX-1 vector.

E.coli BL21(DE3)pLysS (Studier *et al.*, 1990) was transformed with pEX-MR1 or pGEX-MR9, and the relevant products were purified essentially according to the method described by Sambrook *et al.* (1989). When the cell lysates were centrifuged, most of the recombinant proteins were found in inclusion bodies of the precipitate. After successive washing with H_2O and 0.1 M Tris–HCl (pH 8.5) containing 2 M urea, the precipitates were dissolved in a solubilizing buffer [50 mM Tris–HCl (pH 8.0), 1 mM EDTA, 100 mM NaCl containing 8 M urea and 0.1 mM APMSF]. The G-CSF receptor polypeptides were purified by gel filtration on an AcA 54 column which was equilibrated with the solubilizing buffer, and dialyzed against phosphate-buffered saline (PBS). The purified proteins, which were >90% pure, were used to immunize rabbits to obtain the antisera.

Immunoblot analysis

About 2×10^7 cells collected by centrifugation were suspended in 100 μl of PBS containing a mixture of protease inhibitors (Fukunaga *et al.*, 1990b), lysed by adding an equal vol of 2× sample buffer [0.125 M Tris–HCl (pH 6.8), 4% SDS, 20% glycerol, 5% 2-mercaptoethanol] and sonicated to shear genomic DNA. After heating at 95°C for 5 min, the samples were electrophoresed on a 4–20% gradient polyacrylamide gel. Electroblotting of proteins to a GVHP membrane filter (Millipore) was performed as described previously (Fukunaga *et al.*, 1990b). The blotted filter was rinsed with Block Ace (Dainippon Seiyaku, Co., Japan) for 60 min at 37°C, washed three times with PBS and once with TPBS (PBS containing 0.1% Tween 20), and incubated with 10 ml of TPBS containing 10% Block Ace and 1 μl of anti-MR1 serum or 0.1 μl of anti-MR9 serum for 60 min at room temperature. The filter was then washed three times with TPBS and incubated with 10 ml of TPBS containing 10% Block Ace and 18.5 kBq/ml ^{125}I-labeled F(ab')$_2$ fragment of donkey anti-rabbit Ig antibody (Amersham). After incubation for 60 min at room temperature, the filter was washed six times for 10 min each with TPBS, dried and subjected to autoradiography.

Acknowledgments

We thank Dr S.K.Coleman for proofreading the manuscript, and M.Ikeda for her secretarial assistance. We are grateful to Drs M.Hatakeyama and T.Taniguchi for mouse BAF-B03 and CTLL-2 cell lines, and to Dr H.Matsui for human IL-2. We also thank Dr F.W.Studier for *E.coli* BL21(DE3)pLys and the detailed protocol prior to publication. This work was supported in part by grants-in-aid from the Ministry of Education, Science and Culture of Japan.

References

Bass,S.H., Mulkerrin,M.G. and Wells,J.A. (1991) *Proc. Natl. Acad. Sci. USA*, **88**, 4498–4502.
Bazan,J.F. (1990a) *Immunol. Today*, **11**, 350–354.
Bazan,J.F. (1990b) *Proc. Natl. Acad. Sci. USA*, **87**, 6934–6938.
Bishayee,S., Majumdar,S. Khire,J. and Das,M. (1989) *J. Biol. Chem.*, **264**, 11699–11705.
Cleveland,J.L., Dean,M., Rosenberg,N., Wang,J.Y. and Rapp,U.R. (1989) *Mol. Cell. Biol.*, **9**, 5685–5695.
Cunningham,B.C. and Wells,J.A. (1989) *Science*, **244**, 1081–1085.
D'Andrea,A.D., Lodish,H.F. and Wong,G.G. (1989) *Cell*, **57**, 277–285.
Dexter,T.M., Garland,J, Scott,D., Scolnick.E. and Metcalf,D. (1980) *J. Exp. Med.*, **152**, 1036–1047.
Fukunaga,R., Ishizaka-Ikeda,E., Seto,Y. and Nagata,S. (1990a) *Cell*, **61**, 341–350.
Fukunaga,R., Ishizaka-Ikeda,E. and Nagata,S. (1990b) *J. Biol. Chem.*, **265**, 14008–14015.
Fukunaga,R., Seto,Y., Mizushima,S. and Nagata,S. (1990c) *Proc. Natl. Acad. Sci. USA*, **87**, 8702–8706.
Goodwin,R.G. *et al.* (1990) *Cell*, **60**, 941–951.
Hatakeyama,M., Tsudo,M., Minamoto,S., Kono,T., Doi,T., Miyata,T., Miyasaka,M. and Taniguchi,T. (1989a) *Science*, **244**, 551–556.
Hatakeyama,M., Mori,H., Doi,T. and Taniguchi,T. (1989b) *Cell*, **59**, 837–845.
Hayashida,K., Kitamura,T., Gorman,D.M., Arai,K.-I., Yokota,T. and Miyajima,A. (1990) *Proc. Natl. Acad. Sci. USA*, **87**, 9655–9659.
Heldin,C.-H., Ernlund,A., Rorsman,C. and Ronnstrand,L. (1989) *J. Biol. Chem.*, **264**, 8905–8912.
Hibi,M., Murakami,M., Saito,M., Hirano,T., Taga,T. and Kishimoto,T. (1990) *Cell*, **63**, 1149–1157.
Isfort,R.J. and Ihle,J.N. (1990) *Growth Factors*, **2**, 213–220.
Isfort,R.J., Huhn,R.D., Frackleton,A.R., and Ihle,J.N. (1988) *J. Biol. Chem.*, **263**, 19203–19209.
Itoh,N., Yonehara,S., Schreurs,J., Gorman,D.M., Maruyama,K., Ishii,A., Yahara,I., Arai,K. and Miyajima,A. (1990) *Science*, **247**, 324–327.
Katoh,K., Takahashi,Y., Hayashi,S. and Kondoh,H. (1987) *Cell Struct. Function.*, **12**, 575–580.
Koeffler,H.P., Gasson,H. and Tobler,A. (1988) *Mol. Cell. Biol.*, **8**, 3432–3438.
Koyasu,S., Tojo,A., Miyajima,A., Akiyama,T., Kasuga,M., Urabe,A., Schreurs,J., Arai,K., Takaku,F. and Yahara,I. (1987) *EMBO J.*, **6**, 3979–3984.
Metcalf,D. (1989) *Nature*, **339**, 27–30.
Metcalf,D. and Nicola,N.A. (1985) *Leukemia Res.*, **1**, 35–50.
Mizushima,S. and Nagata,S. (1990) *Nucleic Acids Res.*, **18**, 5322.
Morstyn,G., Lieschke,G.J., Sheridan,W., Layton,J. and Cebon,J. (1989) *Trends Pharmacol. Sci.*, **10**, 154–159.
Mosley,B. *et al.* (1989) *Cell*, **59**, 335–348.
Nagata,S. *et al.* (1986a) *Nature*, **319**, 415–418.
Nagata,S., Tsuchiya,M., Asano,S., Yamamoto,O., Hirata,Y., Kubota,N., Oheda,M., Nomura,H. and Yamazaki,T. (1986b) *EMBO J.*, **5**, 575–581.
Nagata,S. (1990) In Sporn,M.B. and Roberts,A.B. (eds), *Peptide Growth Factors and Their Receptors*. Handbook of Experimental Pharmacology, Vol. 95/I , pp. 699–722.
Nara,N., Suzuki,T., Nagata,K., Tohda,S., Yamashita,Y., Nakamura,Y., Imai,Y., Morio,T. and Minamihisamatsu,M. (1990) *Jpn. J. Cancer Res.*, **81**, 625–631.
Nicola,N.A. (1989) *Annu. Rev. Biochem.*, **58**, 45–77.
Nicola,N.A. and Peterson,L. (1986) *J. Biol. Chem.*, **261**, 12384–12389.
Nishizawa,M. and Nagata,S. (1990) *Mol. Cell. Biol.*, **10**, 2002–2011.
Park,L.S., Waldron,P.E., Friend,D., Sassenfeld,H.M., Price,V., Anderson,D., Cosman,D., Andrews,R.G., Bernstein,I.D. and Urdal,D.L. (1989) *Blood*, **74**, 56–65.
Potter,H., Weir,L. and Leder,P. (1984) *Proc. Natl. Acad. Sci. USA*, **81**, 7161–7165.
Sambrook,J., Fritsch,E.F. and Maniatis,T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd edition. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.
Santoli,D., Yang,Y.-C., Clark,S.C., Kreider,B.L., Caracciolo,D. and Rovera,G. (1987) *J. Immunol.*, **139**, 3348–3354.
Seelentag,W.K., Mermod,J.-J., Montesano,R. and Vassalli,P. (1987) *EMBO J.*, **6**, 2261–2265.

Souyri,M., Vigon,I., Penciolelli,J.F., Heard,J.M., Tambourin,P. and Wendling,F. (1990) *Cell*, **63**, 1137–1147.
Spaargaren,M., Defize,L.H.K., Boonstra,J. and de Laat,S.W. (1991) *J. Biol. Chem.*, **266**, 1733–1739.
Studier,F.W., Rosenberg,A.H., Dunn,J.J. and Dubendorf,J.W. (1990) *Methods Enzymol.*, **185**, 60–89.
Tsuchiya,M., Asano,S., Kaziro,Y. and Nagata,S. (1986) *Proc. Natl. Acad. Sci. USA*, **83**, 7633–7637.
Tsuchiya,M., Nomura,H., Asano,S., Kaziro,Y. and Nagata,S. (1987) *EMBO J.*, **6**, 611–616.
Uetsuki,T., Naito,A., Nagata,S. and Kaziro,Y. (1989) *J. Biol. Chem.*, **264**, 5791–5798.
Weinstein,Y., Ihle,J.N., Lavu,S. and Reddy,E.P. (1986) *Proc. Natl. Acad. Sci. USA*, **83**, 5010–5014.

Received on April 29, 1991; revised on June 12, 1991